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Muscarinic Supersensitivity and Impaired Receptor Desensitization in G Protein–Coupled Receptor Kinase 5–Deficient Mice

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Summary

G protein–coupled receptor kinase 5 (GRK5) is a member of a family of enzymes that phosphorylate activated G protein–coupled receptors (GPCR). To address the physiological importance of GRK5-mediated regulation of GPCRs, mice bearing targeted deletion of the GRK5 gene (GRK5-KO) were generated. GRK5-KO mice exhibited mild spontaneous hypothermia as well as pronounced behavioral supersensitivity upon challenge with the nonselective muscarinic agonist oxotremorine. Classical cholinergic responses such as hypothermia, hypoactivity, tremor, and salivation were enhanced in GRK5-KO animals. The antinociceptive effect of oxotremorine was also potentiated and prolonged. Muscarinic receptors in brains from GRK5-KO mice resisted oxotremorine-induced desensitization, as assessed by oxotremorine-stimulated [^{35}S]GTPγS binding. These data demonstrate that elimination of GRK5 results in cholinergic supersensitivity and impaired muscarinic receptor desensitization and suggest that a deficit of GPCR desensitization may be an underlying cause of behavioral supersensitivity.

Introduction

Many important neuromodulators function through activation of specific pre- and postsynaptic G protein–coupled receptors (GPCRs). These include the adrenergic, muscarinic, serotonergic, and dopaminergic systems, as well as a great variety of peptidergic systems. Binding of such substances to their specific cell surface receptors leads to activation of the intracellular G protein–mediated effector pathways, such as adenylyl cyclase, phospholipase C-β, or various ion channels, which then alter neuronal responsiveness (Dickey and Birnbaumer, 1993). One notable feature of these G protein pathways is that the cell surface receptors are subject to precise regulation of their sensitivity. Prolonged or repeated exposure of a receptor to its agonist leads to diminished responsiveness of the receptor to further stimulation, or desensitization (Hausdorff et al., 1990).

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One important mechanism for receptor desensitization is the uncoupling of the activated receptors from further stimulation of their G proteins. This form of desensitization is mediated by the phosphorylation of the activated receptor by members of the family of G protein–coupled receptor kinases (GRKs) (Premont et al., 1995; Pitcher et al., 1998). GRK-phosphorylated receptors bind to an arrestin protein, which prevents the receptor from activating more G proteins despite the continued binding of agonist (Pitcher et al., 1998). Six distinct GRK genes are known, named GRK1 through GRK6, which can be classified into three distinct groups based on gene structure, sequence similarity, function, and regulation (Premont et al., 1995). Several GRK enzymes are found widely throughout the body and in the brain, but the relative importance of each GRK in any tissue is presently unclear (Pitcher et al., 1998).

GRK5 is the best-characterized member of the GRK4 subfamily of GRKs, which also contains GRK4 and GRK6 (Premont et al., 1995). The GRK5 mRNA is expressed widely in brain and peripheral tissues, with the highest expression evident in heart, lung, and placenta; however, the expression pattern of the GRK5 protein is not well characterized. In model systems, GRK5 has been shown to be able to phosphorylate several distinct GPCR types, including rhodopsin, β_2-adrenergic, M_2-muscarinic, secretin, endothelin ET_1, angiotensin AT_1, and thyroid-stimulating hormone receptors (Inglese et al., 1993; Kunapuli et al., 1994; Premont et al., 1994; Menard et al., 1996; Pitcher et al., 1998; Tsuga et al., 1998). Overexpression of GRK5 in the heart revealed that the exogenous GRK5 can augment desensitization of the β-adrenergic–mediated but not the angiotensin II–mediated contractile responses (Rockman et al., 1996). However, physiological substrate receptors for endogenous GRK5, and the importance of GRK5 for maintaining normal physiological responses, remain unknown.

To examine the function of GRK5, the mouse GRK5 gene was inactivated by homologous recombination. Mice lacking functional GRK5 were then assessed for their ability to adapt when challenged with GPCR agonists. Strikingly, these animals are supersensitive to challenge with the muscarinic agonist oxotremorine, and display an apparent lack of desensitization of muscarinic receptor responsiveness.

Results

Generation of GRK5-KO Mice

To begin to address the functions of GRK5 in vivo, the GRK5 gene was targeted by homologous recombination in embryonic stem (ES) cells, as shown in Figure 1A. The targeting construct introduced two loxP sites into the gene, flanking exons 7 and 8, as well as a selectable marker gene cassette also flanked by loxP sites. Transient transfection of a targeted ES cell clone with cre recombinase led to loss of the sequences between the loxP sites, leaving a deleted GRK5 gene (GRK5-KO) with

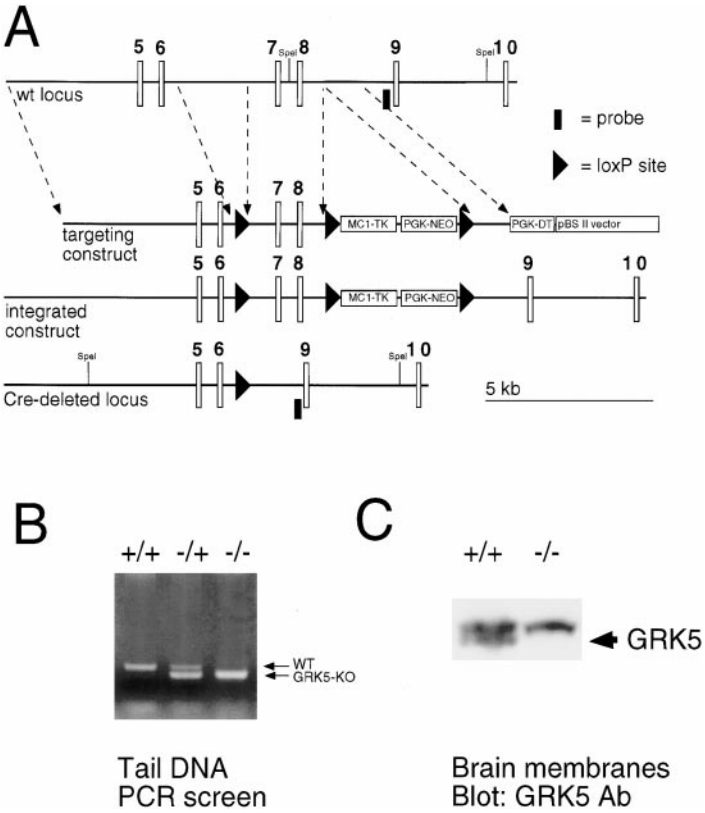

Figure 1. Targeted Inactivation of the Mouse GRK5 Gene

(A) Schematic diagram of the wild-type GRK5 locus, the GRK5/lox targeting vector, the integrated targeting construct, and the Cre recombinase-deleted GRK5 locus (GRK5-KO). GRK5 exons are shown as open boxes and numbered from the first coding exon according to Premont et al. (1999). LoxP sites are shown as filled triangles, and the location of the Southern blot probe as a filled box. Relevant SpeI restriction sites are indicated.

(B) Genotyping of targeted GRK5-KO mice. The wild-type and GRK5-KO loci were distinguished by triplex PCR amplification. The wild-type GRK5 locus gives a 410 bp band, while the GRK5-KO locus gives a 315 bp band, as indicated by the arrows.

(C) GRK5 protein expression by Western blotting. Brain proteins from wild-type and GRK5-KO animals were subjected to immunoblotting using an anti-GRK5 antiserum (Oppermann et al., 1996). GRK5-KO homozygote animals exhibit a loss of the 67 kDa immunoreactive band compared with wild-type animals (arrow). Similar blots were also obtained with brainstem, hypothalamus, and striatum (data not shown). The 68 kDa band may represent the closely related GRK6 or GRK4 protein, which cross-reacts with this antiserum.

no exons 7 and 8, and no inserted marker genes. Exons 7 and 8 encode critical subelements I through III of the protein kinase catalytic domain (Hanks et al., 1988). The GRK5-KO gene contains intact exons 1–6, and could potentially produce a transcript encoding amino acids 1–178, but exon 9 would be spliced out of frame and lead to termination after the addition of 16 novel residues.

Proper targeting of the GRK5 gene, and subsequent animal genotyping, was assessed using triplex polymerase chain reaction (PCR) amplification (Figure 1B). Western blot analysis of GRK5 protein expression in brain membranes reveals the presence of GRK5 immunoreactivity in wild-type mice but not in the GRK5-KO animals (Figure 1C). The upper band may represent the GRK4 or GRK6 subtypes that are also recognized by this monoclonal antibody.

Initial Characterization of GRK5-KO Mice

The homozygote GRK5-KO mice are viable and present no gross anatomical differences nor striking behavioral abnormalities. In locomotor activity tests, knockout mice were not different from wild-type littermates in horizontal and vertical activities nor stereotype scores (data not shown). Classical locomotor responses (Wilcox et al., 1980; Wang et al., 1997) to the D1/D2 dopamine receptor agonist apomorphine (see Figure 3B) and cocaine (data not shown) also did not differ between GRK5-KO and littermate controls. The only intrinsic difference detected between untreated GRK5-KO and wild-type animals was in core body temperature, which was ~0.9°C lower in GRK5-KO mice (basal core temperature was 37.3 ± 0.09°C , n = 30, in wild-type animals and 36.4 ± 0.1°C, n = 28, in GRK5-KO mice).

Behavioral Responses to Muscarinic Agonist

The central cholinergic system is known to be involved in thermoregulation (Lomax, 1970). Muscarinic agonist treatment produces rapid and profound hypothermia, and this response has been used classically to assess muscarinic receptor sensitivity in vivo (Dilsaver and Alessi, 1988). In light of the subtle differences in core temperatures, we challenged mice with the nonselective muscarinic agonist oxotremorine (Gomeza et al., 1999) to assess potential changes in hypothermic parameters in the GRK5-KO mice. In this paradigm, the GRK5-KO mice exhibited a more pronounced and longer lasting depression in body temperature than did wild-type control mice (Figure 2A). In contrast, central hypothermic responses to the serotonin 5-HT1A receptor agonist 8-OH-DPAT (Overstreet et al., 1998) did not differ between genotypes (Figure 2B).

It is well established that activation of central muscarinic pathways results in a characteristic set of manifestations, known as cholinergic syndrome. These behaviors include hypoactivity, tremor, and salivation, as well as hypothermia. All of these behaviors are considered to be dependable measures of the responsiveness of central muscarinic receptors (Dilsaver and Alessi, 1988; Gomeza et al., 1999). Oxotremorine treatment produced more potent and longer lasting depression of horizontal activity in mutant versus wild-type mice (Figure 3A). Notably, the dopamine receptor agonist, apomorphine, produced similar climbing responses in both genotypes (Figure 3B). Further, mice challenged with oxotremorine exhibited increased and prolonged tremor (Figure 4A) and salivation (Figure 4B). Thus, GRK5-KO mice exhibit

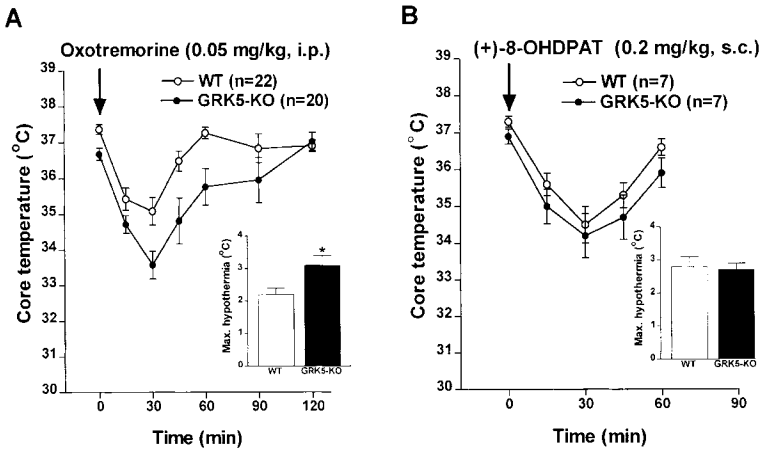

Figure 2. Hypothermic Responses to Muscarinic and Serotonergic Agonists in Mutant and Wild-Type Mice

(A) Effect of oxotremorine (0.05 mg/kg, i.p.) on body temperature. (Inset) Averaged drop in body temperature (maximal hypothermia) measured 30 min after drug administration.

(B) Effect of 5HT1A receptor agonist ((+)-8-OHDPAT, 0.2 mg/kg, subcutaneously) on body temperature. (Inset) Averaged drop in body temperature (maximal hypothermia) measured 30 min after drug administration. Means ± SEM are shown. *Significantly different from wild-type (WT) mice, $P < 0.05$, Student's *t*-test. Note that there is no significant difference between genotypes in hypothermic response to (+)-8-OHDPAT.

increased sensitivity to a spectrum of responses mediated by muscarinic cholinergic receptors.

Muscarinic Agonist-Mediated Analgesia in GRK5-KO Mice

Another well established manifestation of central muscarinic receptor stimulation is antinociception. Muscarinic agonists produce potent analgesia in tests assessing central pain perception. To examine the effects of GRK5 deletion on pain perception (Hartvig et al., 1989), mice were challenged with oxotremorine and tested in the hot-plate test (Konig et al., 1996; Gomeza et al., 1999). Mice of either genotype, when injected with vehicle, responded equally rapidly when placed on the hot plate (Figure 5A). Oxotremorine-treated GRK5-KO mice, however, responded to high temperature significantly less well than did the wild-type control animals. Moreover, these antinociceptive responses persisted longer (Figure 5A) and were detected at a lower dose of oxotremorine (Figure 5B) than in the wild-type mice. Mice of different genotypes did not differ in their basal response to the hot plate (wild type: 6.06 ± 0.28 s; GRK5-KO: 6.04 ± 0.23 s).

To test whether the observed behavioral supersensitivity of GRK5-KO mice is reflective of altered muscarinic receptor signaling, and does not represent altered pharmacokinetic or pharmacodynamic properties of oxotremorine, we also treated mice with physostygmine, which raises endogenous acetylcholine levels by inhibiting acetylcholinesterase (Galeotti et al., 1996). Physostygmine (0.2 mg/kg, intraperitoneally [i.p.]) produced significant analgesia in both GRK5-KO and wild-type mice, but continued to invoke analgesia in GRK5-KO mice even 90 min after injection, by which time the responsiveness of wild-type mice had returned to basal levels (data not shown). These studies reveal that regardless of the means of receptor stimulation, either directly by exogenous agonist or indirectly by preventing degradation of endogeneous acetylcholine, the analgesic effects of muscarinic receptor stimulation in GRK5-KO mice are augmented and prolonged.

Impaired Muscarinic Receptor Desensitization in GRK5-KO Mice

The sensitivity of GRK5-KO mice to muscarinic agonists suggests that there may be changes in either neuronal muscarinic receptor number or responsiveness. There were no differences in the number of oxotremorine binding sites nor in the affinity of the receptor in wild-type

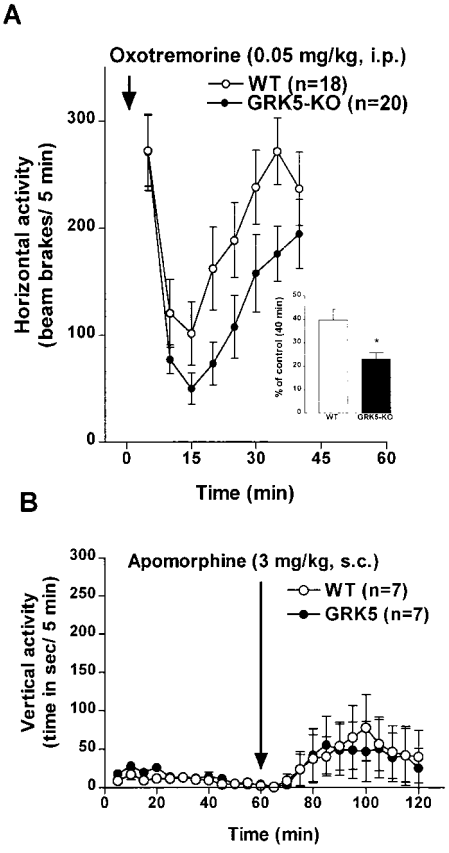

Figure 3. Locomotor Response to Muscarinic and Dopaminergic Agonists in Mutant and Wild-Type Mice

(A) Effect of oxotremorine (0.05 mg/kg, i.p.) on horizontal activity. (Inset) Overall decrease in activity (total horizontal activity counts for 40 min in saline-treated wild-type mice: 3997 ± 384, n = 11; in mutant mice: 4787 ± 516, n = 10) during 40 min after drug administration. Means ± SEM are shown. *Significantly different from wild-type mice, $P < 0.05$, Student's *t*-test.

(B) Effect of apomorphine (3.0 mg/kg, subcutaneously) on vertical activity. Note that there is no significant difference between genotypes in climbing response to apomorphine.

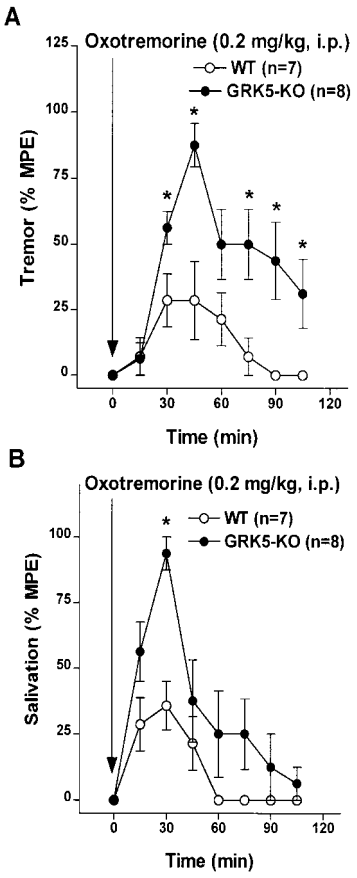

Figure 4. Tremor and Salivation in Mutant and Wild-Type Mice

(A) Tremor and (B) salivation following oxotremorine (0.2 mg/kg, i.p.) in mutant and wild-type mice. Means ± SEM are shown. To determine differences between mouse genotypes, a two-way repeated measure analysis of variance was used and differences were identified using a two-tailed *t*-test contrast matrix. *Significantly different from wild-type mice, $P < 0.05$.

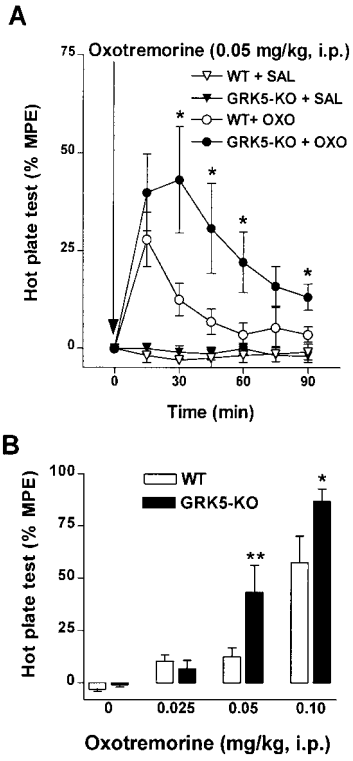

Figure 5. Antinociceptive Effect of Oxotremorine in Hot Plate Test in Mutant and Wild-Type Mice

(A) Time course of oxotremorine (0.05 mg/kg, i.p.) or saline (10 ml/kg, i.p.) induced analgesia (% MPE = % Maximum Possible Effect). To determine differences between mouse genotypes, a two-way repeated analysis of variance was used and differences were identified using a two-tailed *t*-test contrast matrix. Significantly different from wild-type mice, *$P < 0.05$.

(B) Dose response of oxotremorine-induced analgesia 30 min after drug administration. Each set of data was performed on independent groups of 8–12 knockout and 11–15 wild-type animals. Means ± SEM are shown. Significantly different from wild-type mice, *$P < 0.05$, **$P < 0.01$; Student's *t*-test.

and GRK5-KO mouse brain regions (striatum, brainstem, and hypothalamus) as determined by saturation binding of [^{3}H]oxotremorine (Table 1).

Potential changes in receptor sensitivity were assessed indirectly by measuring receptor coupling to G proteins. In this approach, freshly dissociated brain stem pieces were pre-exposed to millimolar concentrations of oxotremorine-M (a more stable analog of oxotremorine) (Birdsall et al., 1978) for 15 min to allow for activation and desensitization of muscarinic receptors (Kunapuli et al., 1994; Bernard et al., 1998). After this pre-exposure period, membranes were prepared and assessed for [^{35}S]GTPγS binding during a 5-min stimulation in the presence of increasing concentrations of oxotremorine-M (Figure 6). Under these conditions, pretreatment with muscarinic agonist completely abolished oxotremorine-stimulated binding of [^{35}S]GTPγS to membranes prepared from wild-type animals, suggesting that these receptors had been desensitized during pretreatment. However, oxotremorine pretreatment did not reduce muscarinic receptor coupling to G proteins in brain membranes from GRK5-KO mice, indicating that without GRK5, these muscarinic receptors are apparently resistant to desensitization. Similar results were seen in striatum either by the same method or ex vivo, where the desensitization of muscarinic receptors was induced in animals by systemic treatment with 0.5 mg/kg oxotremorine (i.p.) 30 min before sacrifice and preparation of striatum membranes (data not shown). By comparison, isoproterenol-stimulated binding of [^{35}S]GTPγS through β-adrenergic receptors could be prevented by pretreatment with isoproterenol in brainstems from both wild-type and GRK5-KO mice (data not shown).

Discussion

Desensitization of GPCRs represents an important step in agonist-dependent receptor regulation (Pitcher et al., 1998). Numerous in vitro studies have demonstrated the fundamental role of this process in cellular functions. However, few studies have been devoted to elucidation of the function of these processes in vivo. The recent availability of transgenic models, particularly the generation of knockout mice, has greatly facilitated evaluation of the physiological role of specific proteins at the level of the whole organism. Genetic deletion of GRK5 has

Table 1. Muscarinic Receptor Binding Sites in Brain Regions of Wild-Type (WT) and KO Mice

	WT		GRK5-KO	
Brain Region	B_{MAX} (fmol/mg)	K_D (nM)	B_{MAX} (fmol/mg)	K_D (nM)
Hypothalamus	101 ± 15	6.5 ± 2.7	117 ± 11	8.3 ± 2.0
Brainstem	101 ± 20	5.2 ± 0.9	94 ± 11	5.4 ± 0.8
Striatum	91 ± 10	3.3 ± 0.1	91 ± 19	3.3 ± 0.6

Binding was performed on membranes from different brain regions with increasing concentrations of ^{3}H-oxotremorine-M (0–12 nM). Nonspecific binding was determined with 10 μM unlabeled oxotremorine-M. Data are the mean ± SEM of three experiments performed in duplicate.

been used here to gain insight into the functional role of this regulatory protein. This approach is particularly valuable, since there are no useful pharmacological modulators of this protein known to date.

From numerous in vitro studies, it is known that GRK5 is involved in phosphorylation of several GPCRs, including subtypes of muscarinic receptors (Inglese et al., 1993; Kunapuli et al., 1994; Premont et al., 1994; Menard et al., 1996; Tsuga et al., 1998). A loss of GRK5 would be expected to result in decreased receptor phosphorylation, which appears to be critically important for receptor desensitization following agonist stimulation (Pitcher et al., 1998). However, previously it has not been determined whether this will result in enhanced receptor responsiveness in vivo or which receptor populations are affected. The present observations unequivocally demonstrate that not only are muscarinic receptors resistant to desensitization in the GRK5-KO mouse, but they also appear to be supersensitive to muscarinic agonists.

From initial characterization of the GRK5-KO mice, it became evident that under basal, unchallenged conditions, these mice demonstrate a very modest phenotype, because only a slight decrease in body temperature was found. This observation of a weak phenotype should not be interpreted to suggest that GRK5 function is unimportant in biological processes, but rather that it plays little role in setting the basal tone of the signaling pathway. In a transgenic animal that has lost a component that is involved in dampening a stimulatory signal, no effect may be seen until that signal is provided. Upon challenge with the relevant agonist, the animal is no longer able to compensate to the same degree as his wild-type littermate. It is also possible that there may be functional substitutes for the missing GRK5 that allow the animal to maintain a normal phenotype. For example, other GRKs or downstream signaling elements such as protein kinase A may be involved in regulating the receptors (Hausdorff et al., 1990). However, the apparent inability of brain muscarinic receptors to desensitize in GRK5-KO animals argues that such compensation is minimal. Further, no obvious increases in the levels of other GRKs in the brain are apparent by Western blotting (data not shown).

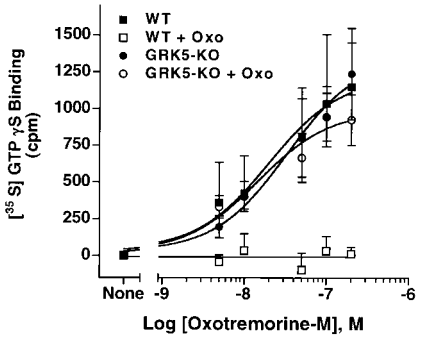

Figure 6. [^{35}S]GTPγS Binding to Brainstem Membranes from Mutant and Wild-Type Mice

Brainstem pieces were incubated at 37°C, 15 min prior to membrane preparation in the presence or absence of 5 mM oxotremorine-M. [^{35}S]GTPγS binding to membranes was determined after 5 min stimulation with 0–200 nM oxotremorine-M. Total [^{35}S]GTPγS binding is portrayed after subtracting unstimulated [^{35}S]GTPγS binding from each point; data were analyzed by nonlinear regression using GraphPad Prism software. In the absence of agonist stimulation, basal [^{35}S]GTPγS binding: wild-type mice, 2748 ± 143; GRK5-KO, 2707 ± 573 cpm. Oxotremorine pretreated wild-type brainstems did not converge to the nonlinear regression analysis, indicating that no oxotremorine-M concentration-dependent stimulation of [^{35}S]GTPγS binding occurs during the 5 min agonist treatment.

Behavioral analyses were performed after challenging animals with a number of agonists to seek out the specific receptors affected by the loss of GRK5. Remarkably, we found no differences in cocaine-induced locomotor responses and climbing responses following high doses of apomorphine, a direct D1/D2 dopamine receptor agonist (Wilcox et al., 1980). Similarly, hypothermic responses to stimulation of serotonin 5-HT1A receptor by 8-OH DPAT (Overstreet et al., 1998) did not differ between the genotypes. These data suggest that the relative responsiveness of dopamine receptors, affected by cocaine and apomorphine, as well as the 5-HT1A subtype of serotonin receptors, seems to be unchanged by deletion of GRK5.

Nonetheless, the GRK5-KO mice were found to be notably hypersensitive to behavioral effects of the nonselective muscarinic agonist oxotremorine. The classical responses to muscarinic stimulation, such as hypothermia, tremor, salivation, and locomotor suppression (Lomax, 1970; Saligaut et al., 1985; Dilsaver and Alessi, 1988; Janowsky et al., 1994; Overstreet et al., 1998; Gomeza et al., 1999) were enhanced and prolonged in mutant mice in comparison with littermate controls. Further, the antinociceptive effect of oxotremorine was also significantly potentiated. The lack of selective agents to evaluate receptor subtype specificity precludes definitive clarification of the muscarinic receptor subtype involved. However, a recent report on M2 muscarinic receptor knockout mice strongly suggests that the vast majority of the behaviors evaluated here may be mediated by this receptor subtype (Gomeza et al., 1999). Therefore, behavioral supersensitivity to oxotremorine in GRK5-KO mice most likely reflects enhanced responsiveness subsequent to impaired desensitization of M2

muscarinic receptors. However, these data do not preclude the possibility that other muscarinic receptors and/or other GPCRs could be affected in these mice. In addition, since M2 receptors are abundant in the cardiovascular system, future studies in these animals should be useful to evaluate the role of GRK5 in controlling cardiac responses.

Six GRKs have been identified to date, while more than 1500 GPCRs have already been cloned (Pitcher et al., 1998). Thus, redundancy in the mechanisms of GPCR regulation, such as the involvement of one GRK in the phosphorylation of multiple receptors, is to be expected. However, the large effect seen in muscarinic responsiveness in the GRK5-KO animals argues that remarkably distinct GPCR/GRK specificity does exist in vivo. Establishing the portfolio of receptors regulated by each GRK in vivo is one of the attractive applications of GRK-KO animals. To date, it has been demonstrated that supersensitivity to olfactory stimuli (Peppel et al., 1997) and muscarinic cholinergic airway responsiveness (Walker et al., 1999) is present in GRK3-KO animals, that cardiac supersensitivity to β-adrenergic stimulation is present in heterozygous GRK2-KO mice (Jaber et al., 1996; Rockman et al., 1998), and that supersensitivity to light is present in GRK1-KO animals (Chen et al., 1999). Other altered receptor responses are almost certain to be found in these various GRK-deficient animals.

It is reasonable to postulate that apparent sensitization of a receptor response may reflect an actual loss of desensitization of the receptor. A lack of oxotremorine-mediated desensitization in GRK5-KO mice was demonstrated biochemically in the [^{35}S]GTPγS binding assay. In wild-type animals, a 15 min pretreatment with a high dose of oxotremorine desensitized the receptors, preventing [^{35}S]GTPγS binding in response to a second addition of oxotremorine. However, in GRK5-KO mouse brain stem, oxotremorine pretreatment did not impede oxotremorine-stimulated [^{35}S]GTPγS binding to membrane G proteins. This was true whether the receptor was exposed to oxotremorine in vivo or in vitro. These observations suggest that muscarinic receptors in GRK5-KO mice are resistant to desensitization, which is most likely due to the loss of GRK5. It is important to note that this altered desensitization was not accompanied by a measurable change in receptor number, as revealed by binding studies. Taken together with the behavioral data, this suggests that the apparent behavioral sensitization to muscarinic agonists may be due to apparent loss of receptor desensitization.

Overstimulation of muscarinic receptors has been implicated in the pathogenesis of several human conditions, such as depression, stress responses, and multiple chemical sensitivities (Janowsky et al., 1994; Overstreet et al., 1996; Markou et al., 1998; Sapolsky, 1998). Moreover, rodent animal models of behavioral muscarinic supersensitivity have been developed for these conditions (Orpen and Steiner, 1995; Overstreet et al., 1998). It would be of interest to use GRK5-KO mice as test subjects to evaluate the role of GRK5-mediated receptor desensitization in these manifestations. Behavioral sensitization has been suggested to be critically involved in drug dependence, tolerance, and addiction (Nestler and Aghajanian, 1997). The present observations raise the important question as to whether the impairment of GRK-mediated desensitization may contribute to the altered responsiveness of the receptors involved in these conditions, such as dopamine and opioid receptors.

In summary, GRK5-KO mice demonstrate marked behavioral supersensitivity to muscarinic agonist stimulation. Importantly, the observed behavioral supersensitivity is correlated with an apparent lack of muscarinic receptor desensitization in functional biochemical assays. These data demonstrate, in an in vivo animal model, that GRK5-mediated regulation of GPCR signaling is physiologically relevant and that muscarinic receptors are likely in vivo targets for this kinase.

Experimental Procedures

Targeted Deletion of the Mouse GRK5 Locus

To create the Triple-Lox/GRK5 targeting vector, first a set of three Triple-Lox vectors was created as follows. The LoxL vector was prepared from pBS II by creating a new multiple cloning site containing NotI and NheI sites, a loxP element, and HindIII and Sse8387I sites. The LoxC vector was created similarly with a new multiple cloning region containing NotI and Sse8387I sites, a loxP element, SalI, HindIII, and MfeI sites, another loxP element, and AscI and FseI sites. MC1-TK (thymidine kinase) (Mansour et al., 1988) and PGK-NEO (neomycin resistance) (from pD383, kind gift of Rene Hen, Columbia) cassettes were inserted irreversibly between these two loxP elements using the SalI, HindIII, and MfeI sites to create the LoxC/TK-NEO vector. The LoxR vector was created similarly to contain a new multiple cloning site containing NotI, AscI, HindIII, EcoRI, PmeI, MfeI, SalI, and FseI sites, and was further modified by irreversible addition of the PGK-DT (diphtheria toxin) cassette (kind gift of Akira Imamoto, University of Chicago) at the MfeI and SalI sites to create the LoxR/DT vector.

Phage λ carrying fragments of the mouse GRK5 gene from the 129/SVJ strain were obtained and sequenced as described (Premont et al., 1999). The 3-kb HindIII fragment containing exons 7 and 8 (the gene fragment to be flanked by loxP sites, or floxed), and the 5-kb NotI–XbaI fragment containing exons 5 and 6 (long recombination arm) were ligated into the HindIII and NotI–NheI sites of the LoxL vector, respectively. The 1.5-kb HindIII–EcoRI gene fragment from between exons 8 and 9 was ligated into those sites in the LoxR/DT vector. The long recombination arm, loxP site, and gene fragment to be floxed were excised from the LoxL vector with NotI and Sse8387I and ligated into those sites of the LoxC/TK-NEO vector. The long recombination arm, floxed gene fragment, and floxed TK-NEO marker cassette were excised from this vector with NotI and AscI and inserted into those sites in the LoxR/DT vector to create the final targeting construct (Figure 1A).

Growth and selection of targeted ES cells and creation of chimeric mice was performed essentially as described (Hogan, 1994). The targeting DNA was linearized by digestion with NotI and electroporated into AK7 ES cells. Cells were selected for growth in media containing 200 μg/ml G418, and surviving clones were tested for proper integration by amplification of a DNA band from the PGK promoter of the NEO marker gene to a region near exon 9 adjacent to the targeting construct. The identity of positive clones was confirmed by Southern blotting of genomic DNA isolated from the cells and digested with SpeI, using a probe from adjacent to exon 9 (Figure 1A). A targeted cell clone with a normal karyotype was expanded and 10×10^6 cells were transfected with 11.5 μg of pBS II-PGK-NLS/Cre recombinase DNA. The PGK-NLS/Cre plasmid contains the PGK promoter driving transcription of Cre recombinase modified to contain a nuclear localization signal (from pNLS-Cre, a kind gift of Samuel Aparicio, Cambridge, UK). Electroporated cells were plated, and 2 days later split to varying densities, and then selected for growth in the presence of 2 μM gangcyclovir. Surviving clones were tested for deletion of the floxed TK-NEO marker cassette and the floxed GRK5 exon 7 and 8 fragment by amplification and by Southern blotting. A cell clone found to lack both floxed cassettes was microinjected into day 3.5 C57BL/6J mouse blastocysts, which were then injected into the uterus of a day 2.5 pseudopregnant B6SJLF1/J mouse. Chimeric offspring were crossed with

C57BL/6J mice to generate agouti pups that carried the deleted GRK5 gene. Heterozygote animals were interbred to obtain the wild-type and homozygote GRK5-KO animals studied here. Genotyping was routinely performed on tail tip DNA using a PCR method utilizing three primers to simultaneously detect the wild-type and mutant loci (Figure 1B).

Western Blots

Mouse brain regions were dissected on ice, immediately frozen in liquid nitrogen, and stored for less than 1 week at −80°C. Crude membranes were prepared from mouse brain regions by polytron homogenization in sodium phosphate buffer (50 mM; pH 7.4) followed by centrifugation at 20,000 × g for 30 min. Aliquots (60 μg) of each sample were collected and centrifuged (5 min; 14,000 rpm, Eppendorf 5414 centrifuge). Pellets were solubulized in lysing buffer (10 mM Tris [pH 7.4], 5 mM EDTA, protease inhibitor cocktail [Roche Molecular Biochemicals, Indianapolis, IN], 1% Nonidet P-40 [Sigma, St. Louis, MO]) and separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) and blotted with monoclonal anti-GRK5 (A16/17) [Oppermann et al., 1996] followed by anti-mouse–horseradish peroxidase conjugated secondary antibody (Amersham, Piscataway, NJ). Bands were visualized on autoradiographic film after enhanced chemilluminescent development (ECS, Amersham, Piscataway, NJ).

Animal Treatment, Drugs, and Behavior

Littermate wild-type and GRK5-KO 3- to 4-month-old C57BL/6× 129SvJ mice were used in these experiments. Rectal body temperatures were determined using a digital thermometer (TH8, Physitemp, Clifton, NJ). The probe was inserted into the rectum and maintained until the temperature reading stabilized.

Spontaneous open field locomotion and rearing behaviors of littermate wild-type and knockout mice of both genders were measured in an Omnitech digiscan activity monitor (42 cm^2). Activity studies were performed between the hours of 10:00 AM and 2:00 PM. Locomotor activity was measured at 5 min intervals, and cumulative counts were taken for data analysis. To evaluate the effects of oxotremorine on locomotor behavior, oxotremorine or vehicle was injected i.p. immediately prior to testing, and different parameters of locomotor activity were monitored for the following 40 min. Climbing responses to apomorphine were tested in habituated animals (60 min habituation time) by assessing duration of interruption of vertical sensors after subcutaneous administration of the drug (Wang et al., 1997).

Tremor and salivation were measured by assigning a value (0, 1, or 2) to represent the degree of the effect observed (Gomeza et al., 1999). Evaluation of salivation and tremor were performed by a colleague who was blinded to genotype and drug treatment. Scoring for salivation and tremor was designated as follows: 0 = no salivation, no tremor; 1 = moderate salivation (moisture visible only around mouth), moderate tremor (intermittent head or body tremor); 2 = marked salivation (moisture on head, neck, and/or chest), nearly continuous whole body tremor. Scores were then normalized (2 = 100%) and averaged for each time point according to genotype and drug treatment.

Antinociceptive Measures

Antinociceptive responses were measured via hot plate response latency (Gomeza et al., 1999). Time was measured from the time the animal was placed upon the hot plate (56°C) until the animal either licked his fore- or hindpaws or flicked his hindpaws. The most prominent observed response was forepaw licking. To avoid tissue damage, an artificial maximum time for exposure was imposed, which prevented the animal from exposure to the plate for greater than 30 s. Data were analyzed by calculating the maximum possible effect, which was determined by accounting for each individual mouse's basal response as well as the imposed maximum cutoff time using the following calculation:

$$100\% \times [(\text{response time} - \text{basal})/(30\text{ s} - \text{basal})] = \%\ \text{MPE}$$

Oxotremorine-M Binding to Brain Membranes

Crude membranes (30 min, 20,000 × g spin) were prepared as described for Western blot analysis. Pellets were resuspended in the same buffer and protein concentrations (50–100 μg/ tube) were used. Saturation binding assays were performed as previously described (Gillard et al., 1987) with increasing concentrations (0–12 nM) of [^{3}H]oxotremorine-M (88.5 Ci/mmol, New England Nuclear, Boston, MA). Nonspecific binding was determined in the presence of 10 μM oxotremorine-M. Samples were incubated at 25°C for 1 hr and then collected via a Brandel cell harvester onto GF/C filters (presoaked with 0.1% polyethylenimine). Filters were washed three times with ice-cold phosphate buffer (10 mM, pH 7.4). After drying, radioactivity was counted in a liquid scintillation counter.

[^{35}S]GTPγS Binding to Brain Membranes

Brain stems were divided bilaterally. Each half was subjected to a gentle manual dissociation of the tissue into pieces with a razor blade. Tissue pieces were then incubated in the presence or absence of 5 mM oxotremorine-M in 1 ml of assay buffer (50 mM Tris [pH 7.4], 100 mM NaCl, 10 mM $MgCl_2$) per sample for 15 min at 37°C. Samples were placed on ice and homogenized by polytron. Membrane preparation buffer (50 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT) was added to each sample, and crude membranes were prepared by centrifugation at 20,000 × g for 20 min. Membranes were washed three times in the same buffer followed by centrifugation for 5 min at 20,000 × g. Membranes were finally resuspended in assay buffer containing 2 μM GDP, and protein concentrations were adjusted to provide 20 μg of protein per assay tube. [^{35}S]GTPγS binding was performed as described previously (Lazareno, 1999). Increasing concentrations of oxotremorine-M were used in the presence of 100 pM [^{35}S]GTPγS (1250 Ci/mmol, New England Nuclear) and 2 μM GDP. The reaction was terminated after 5 min by rapid filtration over GF/C filters using a Brandel cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with ice-cold 10 mM sodium phosphate buffer (pH 7.4) and then counted in a liquid scintillation counter.

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